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RESEARCH ARTICLE

# Colchicine Induced Variation in Sweet Orange (*Citrus sinensis* (L.) Osbeck) cv. Mosambi

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## Abstract

The sweet orange cultivar Mosambi, valued for its low acidity, high juice yield, and superior antioxidant properties, faces challenges due to its high seed count, which diminishes consumer appeal and complicates juice processing. Induced mutagenesis, particularly through colchicine treatment, offers a promising approach for trait improvement. This study characterized variations in two-year-old non-bearing Mosambi mutants induced by colchicine at concentrations of 0.05, 0.10, 0.15, and 0.20%. Colchi-mutants were evaluated against the wild type (WT) for leaf sclerophylly, gas exchange parameters, and stomatal traits. Dose-independent variations were observed, with the CS-9 mutant (0.05% colchicine) showing significant increases in leaf area, fresh mass, and dry mass (2.30–2.43 fold). Colchi-mutants CS-9 and CS-4 exhibited over 28% higher net photosynthesis (A), while colchi-mutants from 0.05 and 0.15% treatments enhanced stomatal conductance and transpiration. A general reduction in stomatal number was observed, accompanied by an increase in size. These findings provide criteria for selecting desirable mutants for sweet orange improvement programs.

**Keywords:** Colchi-mutant, Leaf area, Photosynthesis rate, Stomata, Stomatal conductance.

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## Introduction

Sweet orange (*Citrus sinensis* (L.) Osbeck) holds a prime position in the citriculture industry of India and is commercially grown in the tropical climate (18–21°N) of Central and Southern India. It occupies an area of 0.18 million hectares with an estimated production of 2.87 million tonnes thus contributing 23.47% to total citrus production in India (Anonymous, 2018). The fruit is in great demand among consumers owing to its juice quality and medicinal attributes which are advocated to sick people as it contains a good amount of vitamin C and soluble sugar. The presence of a large sized and high number of seeds however hinders consumer's acceptability, as crushed seeds are the major cause of bitterness in sweet orange juice (Singh *et al.,* 2022). Seedless or partially seedless citrus varieties are generally preferred by the consumers. Different breeding methods are adopted for the induction of seedlessness including inter-ploidy hybridization between diploid and tetraploid parents for the production of triploids, which are largely seedless. However, the desirable tetraploids, which can be used in such hybridization programs to produce triploids are limited and need to be developed to achieve the objective of seedlessness. Colchicine, a potential chemical agent and mitotic inhibitor, is known for inducing hyperploid and enhancing genetic variability in different crops within a very short period of time and has also been commonly used for inducing hyperploid in breeding lines of citrus. Colchicine is potentially used as a chromosome-doubling agent, and it also induces mutation in plants. The term "colchi-mutants"

refers to mutants induced using colchicine (Ari *et al.,* 2015). The mutagenic effects of colchicine on plant performance have been previously documented by Balkanjieva (1980) and Castro *et al.,* (2003). Differential responses of citrus cultivars to colchicine treatment have been attributed to bud mortality or growth retardation in treated tissues (Barrett, 1974). Fatima *et al.* (2015) reported the mutagenic influence of colchicine on the characteristics of sweet orange and mandarin plants. Genetic variation is a fundamental prerequisite for crop improvement, and the induction of variability through physical or chemical mutagens represents a powerful approach for developing trait-specific varieties. This process enables the effective characterization and utilization of variability within generated populations (Grosser *et al.,* 2014). Traits of interest often arise from morphological and physiological modifications, which serve as a basis for plant improvement (Mallick *et al.,* 2016).

In the present study, colchicine was employed to induce variation in sweet orange (cv. Mosambi), with a focus on assessing alterations in morphological and physiological traits in pre-bearing colchi-mutants. The study also aimed to identify key traits that could function as early-stage markers for mutant selection. These markers are expected to correlate with important genetic traits, such as reduced seed count or enhanced stress tolerance, which are critical for advancing breeding programs.

## Materials and Methods

In this study, two-year-old putative mutants of sweet orange (*Citrus sinensis* cv. Mosambi) developed using various concentrations of colchicine (0.05, 0.10, 0.15, and 0.20%), along with the wild type (WT), were evaluated. The plants were grafted onto *Jatti Khatti* (*Citrus jambhiri* Lush.) rootstock and established at a spacing of 1.5 × 1.5 m in the experimental orchard of the Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi, India. The mutants were coded from CS-0 to CS-20, where CS-0 represented the control (WT), CS-1 to CS-9 corresponded to mutants treated with 0.05% colchicine, CS-10 to CS-14 with 0.10%, CS-15 to CS-17 with 0.15%, and CS-18 to CS-20 with 0.20%. Uniform cultural practices were applied across all plants, and variations in leaf sclerophylly and gas exchange parameters were observed over two consecutive years, 2017 and 2018.

To assess alterations in leaf-related parameters, leaf samples aged 40–45 days were collected from the rainy season flushes in four replicates, with ten leaves sampled per replicate from different canopy orientations. Leaf fresh mass (FM) was recorded immediately after sampling, followed by measurement of leaf area (LA) using a Li-Cor LI-3100 area meter (Li-Cor, USA). The leaves were then oven-dried at 70 ± 1°C until constant weight to determine the dry mass (DM). Leaf physiological parameters viz., specific leaf area (SLA), specific leaf weight (SLW), density of foliar tissue (DFT) and succulency (S) were calculated by the formulae suggested by Ennajeh *et al*., 2010. These included (SLA= LA/ DM: in $cm^2/g$ DW), (SLW = DW/LA: in $g/cm^2$ LA), (D = DW/FW X 1000: in g/kg) and [S = (FW-DW)/LA: in $H_2O/cm^2$].

Leaf anatomical analysis was performed on fully mature, expanded leaves by obtaining epidermal impressions of the abaxial surface using the method outlined by Sampson (1961). Stomatal density was assessed by counting the number of stomata per unit field of view (1000 $\mu m^2$) under 40X magnification, utilizing a Motic BA400 microscope with an integrated digital camera. Stomatal length and width were measured at 40 X magnification using Motic software.

Leaf net photosynthesis (*A*), stomatal conductance (*gs*), intercellular $CO_2$ concentration (*Ci*), and transpiration rate (*E*) were measured on leaves aged 60 to 70 days, following the emergence of the rainy season flush in August. Gas exchange parameters were recorded between 09:30 AM and 11:30 AM (IST) on clear days using the LCi-SD Ultra Compact Photosynthesis System (ADC Bioscientific Ltd., Global House, Hoddesdon, UK). Measurements were taken from four mature leaves per plant, specifically the fourth leaf from the shoot tip, positioned on the exterior canopy in the North, South, East, and West orientations.

The experiment was designed using a completely randomized block design (CRBD) with four replications. Data were analyzed through analysis of variance (ANOVA) using the SAS software package (version 9.3, SAS Institute Inc., USA), followed by Tukey's honest significant difference (HSD) test. Statistical significance was determined at $p \leq 0.05$. Pearson's simple correlation analysis was performed to evaluate the relationships between leaf sclerophylly and stomatal parameters, using SPSS software.

## Results and Discussion

Significant variations in leaf sclerophylly were observed across all colchi mutants (Table 1). Most mutants exhibited increases in leaf area, fresh mass, and dry mass compared to the wild type (WT) except for CS-3, CS-8, CS-11, and CS-14. Among the mutants, CS-9 recorded the highest increase in leaf area, fresh mass, and dry mass, with a 2.30-fold increase compared to WT. Conversely, CS-14 showed a significant decrease in leaf area, by 3.83%, which was statistically similar to CS-3. Leaf succulence in colchi mutants was generally higher than in WT. CS-2 and CS-5 showed a 20.43% increase in succulence, while the minimum succulence was recorded in CS-1, with a 6.98% decrease. Specific leaf area (SLA) decreased significantly in all mutants except CS-2 and CS-9, where SLA was 16.93% higher than WT. The maximum reduction in SLA, at 24.72%, was observed in CS-4. Contrary to the SLA trend, significant increases in specific leaf weight (SLW) and density of foliar tissue (DFT) were recorded in most mutants, except CS-2 and CS-9.

**Table 1:** Variation in leaf sclerophylly and stomata of colchi-mutants in comparison to wild type

| *Treatment* | *Leaf area ($cm^2$)* | *Leaf fresh weight (g)* | *Leaf dry weight (g)* | *Succulency ($mg\ H_2O/cm^2$)* | *Specific leaf area ($cm^2/g$)* | *Specific leaf weight ($g/cm^2$)* | *Density of foliage tissue (g/kg)* | *No. of Stomata/ 1000$\mu m^2$* | *Stomata Length (µm)* | *Stomata Width (µm)* |
|---|---|---|---|---|---|---|---|---|---|---|
| CS-0 | 213.0$^{l}$ | 6.03$^{o}$ | 2.08$^{n}$ | 0.0186$^{hgif}$ | 102.56$^{c}$ | 0.0099$^{j}$ | 344.50$^{j}$ | 2.75$^{a}$ | 13.13$^{hfge}$ | 11.78$^{fge}$ |
| CS-1 | 240.13$^{i}$ | 7.01$^{l}$ | 2.85$^{i}$ | 0.0173$^{k}$ | 84.41$^{l}$ | 0.0119$^{d}$ | 405.69$^{b}$ | 2.25$^{ba}$ | 18.00$^{a}$ | 13.70$^{ba}$ |
| CS-2 | 266.25$^{f}$ | 8.17$^{g}$ | 2.22$^{m}$ | 0.0223$^{a}$ | 119.93$^{a}$ | 0.0084$^{k}$ | 271.64$^{m}$ | 2.75$^{a}$ | 14.60$^{dce}$ | 13.03$^{bdec}$ |
| CS-3 | 206.26$^{n}$ | 6.75$^{m}$ | 2.41$^{k}$ | 0.0211$^{b}$ | 85.58$^{k}$ | 0.0118$^{d}$ | 357.03$^{hg}$ | 2.50$^{ba}$ | 14.08$^{dfce}$ | 12.28$^{fdec}$ |
| CS-4 | 275.99$^{e}$ | 8.66$^{e}$ | 3.58$^{h}$ | 0.0184$^{hgij}$ | 77.20$^{p}$ | 0.0130$^{a}$ | 412.94$^{a}$ | 2.25$^{ba}$ | 14.35$^{dfce}$ | 12.08$^{fgde}$ |
| CS-5 | 236.17$^{j}$ | 8.15$^{g}$ | 2.93$^{f}$ | 0.0221$^{a}$ | 80.61$^{n}$ | 0.0124$^{bc}$ | 359.50$^{g}$ | 2.25$^{ba}$ | 13.10$^{hfge}$ | 11.65$^{fg}$ |
| CS-6 | 264.17$^{f}$ | 8.02$^{h}$ | 2.88$^{gh}$ | 0.0194$^{de}$ | 91.65$^{i}$ | 0.0109$^{fe}$ | 359.52$^{g}$ | 2.00$^{b}$ | 15.03$^{dc}$ | 12.43$^{fbdec}$ |
| CS-7 | 330.38$^{b}$ | 10.12$^{b}$ | 3.42$^{c}$ | 0.0203$^{c}$ | 96.75$^{e}$ | 0.0104$^{ihg}$ | 337.53$^{k}$ | 2.00$^{b}$ | 13.45$^{dfge}$ | 12.03$^{fgde}$ |
| CS-8 | 210.06$^{m}$ | 6.76$^{m}$ | 2.51$^{j}$ | 0.0203$^{c}$ | 83.86$^{l}$ | 0.0121$^{dc}$ | 370.43$^{e}$ | 2.00$^{b}$ | 11.70$^{hi}$ | 7.35$^{i}$ |
| CS-9 | 489.93$^{a}$ | 14.63$^{a}$ | 4.69$^{a}$ | 0.0203$^{c}$ | 104.58$^{b}$ | 0.0096$^{j}$ | 320.18$^{l}$ | 2.25$^{ba}$ | 14.53$^{dfce}$ | 12.08$^{fgde}$ |
| CS-10 | 230.71$^{k}$ | 7.55$^{j}$ | 2.92$^{gf}$ | 0.0201$^{c}$ | 79.15$^{o}$ | 0.0127$^{ba}$ | 385.96$^{c}$ | 2.00$^{b}$ | 15.15$^{bc}$ | 11.13$^{fg}$ |
| CS-11 | 210.64$^{m}$ | 6.26$^{n}$ | 2.35$^{l}$ | 0.0186$^{hgif}$ | 89.54$^{j}$ | 0.0112$^{e}$ | 375.94$^{d}$ | 2.50$^{ba}$ | 14.73$^{dce}$ | 13.33$^{bdac}$ |
| CS-12 | 288.92$^{c}$ | 8.74$^{d}$ | 3.24$^{d}$ | 0.0190$^{dgef}$ | 89.25$^{j}$ | 0.0112$^{e}$ | 370.52$^{e}$ | 2.50$^{ba}$ | 11.80$^{hgi}$ | 9.33$^{h}$ |
| CS-13 | 276.33$^{e}$ | 8.43$^{f}$ | 2.98$^{e}$ | 0.0197$^{dc}$ | 92.81$^{h}$ | 0.0108$^{feg}$ | 353.41$^{i}$ | 2.25$^{ba}$ | 16.73$^{ba}$ | 14.60$^{a}$ |
| CS-14 | 204.84$^{n}$ | 6.06$^{o}$ | 2.18$^{m}$ | 0.0189$^{gef}$ | 94.09$^{g}$ | 0.0106$^{fhg}$ | 359.45$^{g}$ | 2.50$^{ba}$ | 12.88$^{hfg}$ | 11.88$^{fge}$ |
| CS-15 | 280.74$^{d}$ | 7.90$^{i}$ | 2.90$^{gf}$ | 0.0178$^{kj}$ | 96.81$^{e}$ | 0.0103$^{ih}$ | 367.21$^{f}$ | 2.00$^{b}$ | 13.48$^{dfe}$ | 13.43$^{bac}$ |
| CS-16 | 290.48$^{c}$ | 8.10$^{g}$ | 2.89$^{gh}$ | 0.0180$^{ij}$ | 100.69$^{d}$ | 0.0100$^{ij}$ | 356.18$^{hi}$ | 2.25$^{ba}$ | 13.95$^{dfce}$ | 12.15$^{fdec}$ |
| CS-17 | 257.14$^{g}$ | 7.54$^{j}$ | 2.70$^{i}$ | 0.0189$^{gef}$ | 95.32$^{f}$ | 0.0105$^{fhg}$ | 357.76$^{hg}$ | 2.00$^{b}$ | 13.90$^{dfce}$ | 12.45$^{fbdec}$ |
| CS-18 | 248.92$^{h}$ | 7.44$^{k}$ | 2.67$^{i}$ | 0.0192$^{def}$ | 93.32$^{hg}$ | 0.0107$^{fhg}$ | 358.65$^{hg}$ | 2.25$^{ba}$ | 13.85$^{dfce}$ | 11.73$^{fge}$ |
| CS-19 | 276.22$^{e}$ | 8.93$^{c}$ | 3.37$^{c}$ | 0.0201$^{c}$ | 81.91$^{m}$ | 0.0122$^{dc}$ | 377.76$^{d}$ | 2.00$^{b}$ | 13.98$^{dfce}$ | 10.75$^{g}$ |
| CS-20 | 250.02$^{h}$ | 7.03$^{l}$ | 2.49$^{j}$ | 0.0182$^{hij}$ | 100.62$^{d}$ | 0.0100$^{ij}$ | 353.73$^{i}$ | 2.00$^{b}$ | 10.85$^{i}$ | 8.35$^{h}$ |
| LSD ($P \leq 0.05$) | 2.24 | 0.07 | 0.04 | 0.0007 | 0.87 | 0.0005 | 2.88 | 0.54 | 1.67 | 1.35 |

The highest increases in SLW and DFT were noted in CS-4, at 34.02 and 19.86%, respectively. Conversely, the largest decreases were observed in CS-2, with reductions of 14.43% in SLW and 21.15% in DFT.

While variations in morphological characteristics may not fully capture the genetic diversity among individuals, they play a significant role in plant breeding, as both qualitative and quantitative traits are utilized to assess genetic diversity (Wi *et al.,* 2007; Dhakshanamoorthy *et al.,* 2010). In the present study, colchi-mutants exhibited both increasing and decreasing trends in leaf area, fresh mass, and succulence. The observed increase in leaf area may be attributed to mutagen-induced cell growth in the lamina, resulting in notable expansion. Similar findings were reported by El-Nashar and Ammar (2016) in *Calendula officinalis* L. mutants treated with lower colchicine concentrations. Conversely, a reduction in leaf area could be due to cellular damage leading to the degradation of indole-3-acetic acid (IAA) (Moore, 1979) or chromosomal alterations. While chromosomal manipulation was beyond the scope of this study, the possibility of polyploidy cannot be ruled out. The increase in leaf fresh mass and succulency might be explained by the reduced leaf area, which allows for greater moisture retention relative to the leaf's surface area. Similar mutagen-induced changes in leaf morphology following lower doses of colchicine have been reported in various fruit crops, including *Ziziphus jujube* Mill. cv. Zhanhua (Gu *et al.,* 2005), grape (Yang *et al.,* 2006), sour jujube (Shanko, 2017), and cape gooseberry (Kumar *et al.,* 2019).

Significant alterations were recorded in the stomatal characteristics (stomata length, width and number) of colchi mutants (Table 1). In CS-1, stomata length was 37.09% more as compared to WT, while reduced stomata length of 17.36% was recorded in CS-20 and did not differ significantly with CS-3, CS-4, CS-9, CS-16, CS-17, CS-18 and CS-19. Stomata width registered and increase of 23.94% in CS-13, while a reduced stomata width of 37.60% was registered in CS-8. Stomata number was registered maximum in control and CS-2. As compared to control significant reduction in the number of stomata was recorded in CS-6, without any significant difference in the colchi mutants CS-7, CS-8, CS-10, CS-15, CS-17, CS-19 and CS-20 (Figure 1).

Phenotypic and genotypic modifications in mutagenic plants often result from various cellular alterations, including changes in stomatal dimensions (length and width), number, distribution, density, and variations in xylem and phloem cell

numbers. In certain cases, these changes lead to pronounced anatomical differences between wild-type plants and induced tetraploids. Parameters such as stomatal number, size, frequency, and chloroplast count have proven to be reliable tools for identifying ploidy levels and facilitating the preliminary characterization of diploids, polyploids, and other taxa (Tang *et al.,* 2015). The observed variation in stomatal length and width, regardless of mutagenic dose, may be attributed to induced genetic damage, mutation rates, or changes in ploidy levels. Stomatal size is positively correlated with ploidy level, while stomatal density typically decreases as ploidy level increases, as reported in pear (*Pyrus* spp.) by Jia and Chen (1985). A comparable increase in stomatal length with higher ploidy levels was reported in *Actinidia deliciosa* (Przywara *et al.,* 1988). Abdoli *et al.* (2013) observed larger stomata, pollen grains, seeds, and flowers in colchicine-induced tetraploid plants of *Echinacea purpurea* (L.). Similarly, Blasco *et al.* (2015) documented the effects of colchiploidy on stomatal traits in loquat (*Eriobotrya japonica*).

Leaf gas exchange parameters *i.e.,* photosynthetic rate (*A*), transpiration rate (*E*), internal carbon dioxide concentration (*Ci*) and stomatal conductance (*gs*) differed significantly in the colchi mutants summarized in Figure 2 A&B. Photosynthetic rate (*A*) in comparison to the WT was higher in the colchi-mutants except CS-1. An increase of 28.61% in *A* was observed in CS-9 and CS-4. An increase in *gs* and *E* was recorded in colchi-mutants developed at 0.05 and 0.15% colchicine. As compared to WT significant increase of 33.33% in the rate of stomatal conductance (*gs*) was observed in CS-15 (0.15%) and maintained statistical parity with CS-16 (0.15%) and CS-5 (0.05%). Transpiration rate (*E*) was significantly higher by 13.98% in CS-15 with similar statistical values in colchi mutants CS-2, CS-6 and CS-8 (0.05%), while colchi mutant CS-14 (0.10%) transpired 12.95% less than the WT. Comparative analysis of *Ci* value over the WT revealed a rise of 6.04% in CS-19 and CS-18 (0.20%). A decrease of 8.89% in the *Ci* value was observed in CS-10 (0.15%) and was statistically similar to colchi-mutants CS-4, CS-7 (0.05%) and CS-16 (0.15%).

In this study, an increase in photosynthetic activity was observed in the colchi mutants compared to the wild type (WT). The enhanced leaf number and density of foliar tissue (DFT) in the mutants likely facilitated improved gaseous exchange, significantly influencing photosynthesis (Lockhart *et al.,* 1996). The observed increase in transpiration rate in the mutants, particularly at analogous colchicine doses, suggests an associated rise in stomatal conductance.

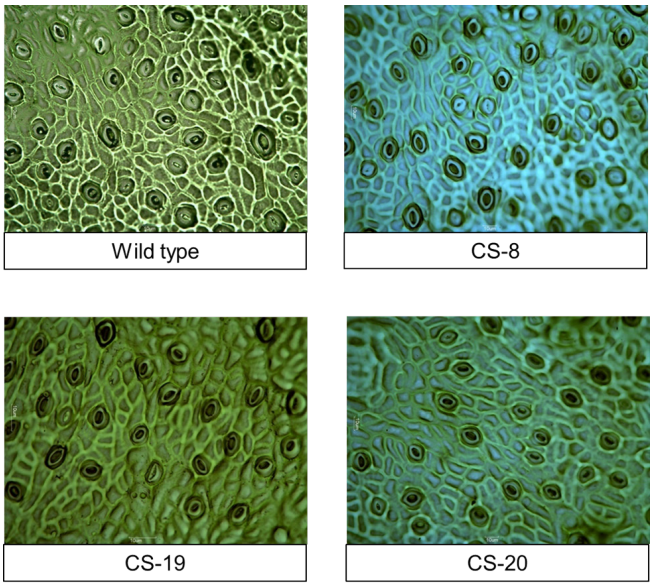


**Figure 1:** Variation in leaf stomata density in the mutants developed through colchicine

**Table 2:** The Pearson correlation of coefficient among the leaf sclerophylly and stomata parameters of colchicine developed Mosambi mutants

| *Parameters* | *Leaf area* | *Fresh weight* | *Dry weight* | *SLA* | *SLW* | *DFT* | *Succulency* | *Stomata Density* | *Length (um)* | *Width (um)* |
|---|---|---|---|---|---|---|---|---|---|---|
| Leaf area | 1 | | | | | | | | | |
| Fresh weight | 0.97** | 1 | | | | | | | | |
| Dry weight | 0.88** | 0.92** | 1 | | | | | | | |
| SLA | 0.34 | 0.21 | -0.14 | 1 | | | | | | |
| SLW | -0.33 | -0.18 | 0.15 | -0.99** | 1 | | | | | |
| DFT | -0.34 | -0.31 | 0.08 | -0.87** | 0.83** | 1 | | | | |
| Succulency | 0.08 | 0.26 | 0.05 | 0.09 | -0.01 | -0.57** | 1 | | | |
| Stomata Density | -0.19 | -0.21 | -0.38 | 0.38 | -0.34 | -0.37 | 0.16 | 1 | | |
| Length (um) | 0.10 | 0.11 | 0.16 | -0.11 | 0.14 | 0.16 | -0.08 | 0.04 | 1 | |
| Width (um) | 0.11 | 0.07 | 0.03 | 0.18 | -0.17 | -0.10 | -0.08 | 0.25 | 0.79** | 1 |

** Correlation is significant at the 0.01 level

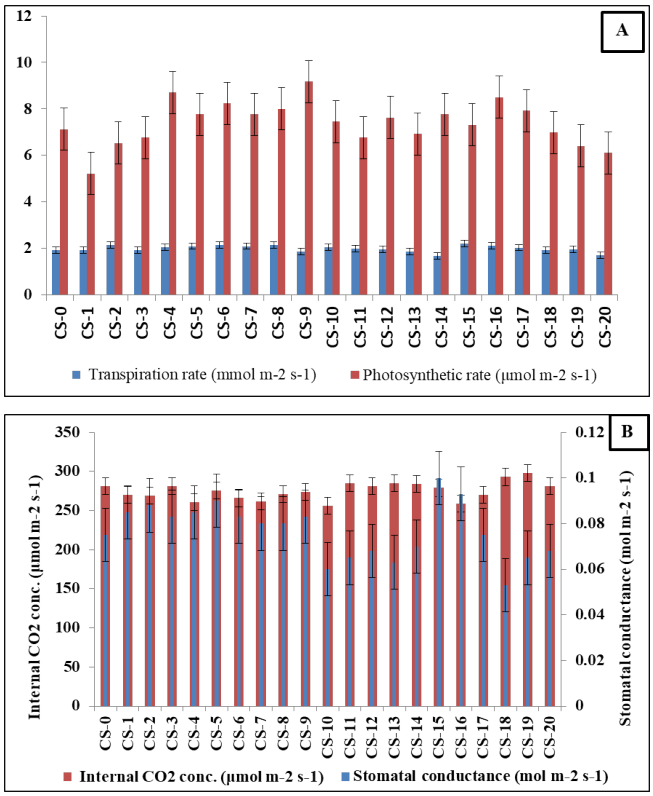


**Figure 2:** (A) Variation in the colchi-mutant's Transpiration rate (*E*) and Photosynthetic rate (*A*) & (B) Internal carbon dioxide concentration (*Ci*) and Stomatal conductance (*gs*)

These findings align with Warner and Edwards (1993), who noted that colchicine-induced polyploidization affects photosynthesis, which is determined by the photosynthetic rate per cell multiplied by the number of cells per unit leaf area. Similarly, Nobel (1999) highlighted the role of stomatal conductance in regulating transpiration rates. Ainsworth *et al.,* (2004) further emphasized that an increase in leaf area contributes to an expanded surface area for gaseous exchange, supporting the results of this study.

The simple correlations of coefficient among the leaf sclerophylly and stomata parameters of putative sweet orange cv. Mosambi mutants along with WT were calculated and presented in Table 2. Leaf area showed a significant positive correlation with the fresh weight (r = 0.97) and dry weight (r = 0.88). Leaf fresh weight was significantly and positively correlated with leaf dry weight (r = 0.92). Specific leaf area showed a complete negative correlation with specific leaf weight (r = -0.99) and density of foliage tissue ( r= -0.87). Specific leaf weight was significantly and positively correlated with the density of foliage tissue (r = 0.83). The density of foliage tissue showed a significant negative correlation with succulency (r = -0.57). Stomata length showed a positive correlation with stomata width (r = 0.79). The other parameters were not significantly correlated for the present set of putative sweet orange cv. Mosambi mutants.

It can be concluded that colchicine treatment has effectively induced significant variations in sweet orange, highlighting its potential for use in sweet orange improvement programs. The observed variations, although independent of colchicine concentration, demonstrated both stimulatory and inhibitory effects on leaf sclerophylly, physiological traits, and anatomical features suggest the possibility of these mutants being a putative tetraploid which however, needs further investigation. Therefore, a comprehensive evaluation of the mutant population across various traits is essential to identify superior mutants for future breeding and improvement initiatives.

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